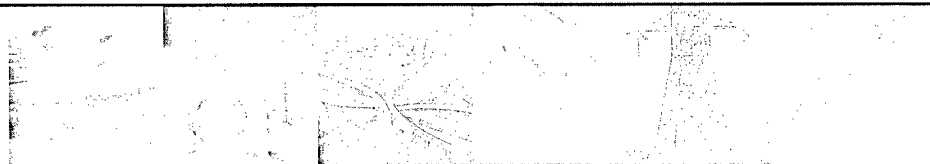


REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE (DD-MM-YYYY) 2004		2. REPORT TYPE Open Literature		3. DATES COVERED (From - To)	
4. TITLE AND SUBTITLE The Application of the Fluoride Reactivation Process to the Detection of Sarin and Soman Nerve Agent Exposures in Biological Samples				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Adams, TK, Capacio, BR, Smith, JR, Whalley, CE, Korte, WD				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) US Army Medical Research Institute of Chemical Defense ATTN: MCMR-UV-PA 3100 Ricketts Point Road Aberdeen Proving Ground, MD 21010-5400				8. PERFORMING ORGANIZATION REPORT NUMBER USAMRICD-P03-026	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) US Army Medical Research Institute of Chemical Defense ATTN: MCMR-UV-RC 3100 Ricketts Point Road Aberdeen Proving Ground, MD 21010-5400				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited					
13. SUPPLEMENTARY NOTES Published in Drug and Chemical Toxicology, 27(1), 77-91, 2004.					
14. ABSTRACT See reprint.					
15. SUBJECT TERMS sarin and soman assay, fluoride reactivation process, carboxylesterase, guinea pigs, monkeys					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UNLIMITED	18. NUMBER OF PAGES 15	19a. NAME OF RESPONSIBLE PERSON Benedict R. Capacio
a. REPORT UNLIMITED	b. ABSTRACT UNLIMITED	c. THIS PAGE UNLIMITED			19b. TELEPHONE NUMBER (include area code) 410-436-1944

DRUG AND CHEMICAL TOXICOLOGY



MARCEL DEKKER
270 MADISON AVE, NEW YORK, NY 10016
www.dekker.com

The Application of the Fluoride Reactivation Process to the Detection of Sarin and Soman Nerve Agent Exposures in Biological Samples[#]

T. K. Adams,¹ B. R. Capacio,^{1,*} J. R. Smith,¹ C. E. Whalley,²
and W. D. Korte¹

¹US Army Medical Research Institute of Chemical Defense, and

²US Army Edgewood Chemical Biological Center,
Aberdeen Proving Ground, Maryland, USA

ABSTRACT

20060126 063

The fluoride reactivation process was evaluated for measuring the level of sarin or soman nerve agents reactivated from substrates in plasma and tissue from in vivo exposed guinea pigs (*Cavia porcellus*), in blood from in vivo exposed rhesus monkeys (*Macaca mulatta*), and in spiked human plasma and purified human albumin. Guinea pig exposures ranged from 0.05 to 44 LD₅₀, and reactivated nerve agent levels ranged from 1.0 ng/mL in plasma obtained from 0.05 LD₅₀ sarin-exposed guinea pigs to an average of 147 ng/g in kidney tissue obtained from two 2.0 LD₅₀ soman-exposed guinea pigs. Positive dose-response relationships were observed in all low-level, 0.05 to 0.4 LD₅₀, exposure studies. An average value of 2.4 ng/mL for reactivated soman was determined in plasma obtained from two rhesus monkeys three days after a 2 LD₅₀ exposure. Of the five types of guinea pig

[#]The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

*Correspondence: Dr. B. R. Capacio, US Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, MD 21010-5400, USA; Fax: (410) 436-1960; E-mail: benedict.capacio@amedd.army.mil.



tissue studied, plasma, heart, liver, kidney and lung, the lung and kidney tissue yielded the highest amounts of reactivated agent. In similar tissue and with similar exposure procedures, reactivated soman levels were greater than reactivated sarin levels. Levels of reactivated agents decreased rapidly with time while the guinea pig was alive, but decreased much more slowly after death. This latter chemical stability should facilitate forensic retrospective identification. The high level of reactivated agents in guinea pig samples led to the hypothesis that the principal source of reactivated agent came from the agent-carboxylesterase adduct. However, there could be contributions from adducts of the cholinesterases, albumin and fibrous tissue, as well. Quantitative analysis was performed with a GC-MS system using selected ion monitoring of the 99 and 125 ions for sarin and the 99 and 126 ions for soman. Detection levels were as low as 0.5 ng/mL. The assay was precise and easy to perform, and has potential for exposure analysis from organophosphate nerve agents and pesticides in other animal species.

Key Words: Sarin and soman assay; Fluoride reactivation; Carboxylesterase; Guinea pigs and monkeys.

INTRODUCTION

Numerous methods have been developed for the determination of animal and human exposure to the highly toxic nerve agents such as tabun (GA), sarin (GB), soman (GD) and VX, and key methods have been reviewed recently.^[1] Those methods include monitoring decreases in blood cholinesterase levels,^[2] residual nerve agent in blood,^[3] hydrolysis products of the nerve agent,^[4-6] fluoride reactivated species,^[7-9] and agent adducts of proteins.^[10] Because the fluoride reactivation process has been shown to be specific, quantitative, retrospective and relatively simple for the analysis of sarin exposures,^[7,8] we have evaluated the application of that process to the analysis of soman and sarin exposures with guinea pigs (*Cavia porcellus*) and rhesus monkeys (*Macaca mulatta*) (hereafter referred to as non-human primates, NHP).

The principal toxic pathway for organophosphorus nerve agents involves the binding of the agent to the serine found at the active site of acetylcholinesterase (AChE).^[11] However, analogous binding with more abundant plasma butyrylcholinesterase (BuChE) can give a greater quantity of substrate for the fluoride reactivation process and improve the sensitivity and precision of the analytical method.^[8] In the reactivation process the fluoride ion cleaves the enzyme from the phosphonyl center and reforms the phosphorus-fluorine bond. In the case of the sarin-BuChE adduct, the cleavage reaction leads to the regeneration or reactivation of the original toxic agent sarin. Unfortunately, the soman-BuChE adduct undergoes rapid aging, which leads to the loss of the pinacolyl group and modification of the structure of the phosphonyl center.^[11,12] Therefore, little, if any, soman would be reactivated.^[10]

However, other protein sources such as carboxylesterase (CaE) and albumin have been observed to form reasonably stable adducts with soman.^[13-18] The soman-CaE adduct has been shown to yield reactivated soman.^[13] This paper describes the analytical utility of these and possibly additional sources of reactivated nerve agent for determining levels of sarin and soman nerve agent exposure.

MATERIALS AND METHODS

Chemicals and Solid Phase Extraction (SPE) Cartridges

Ethyl acetate (99.8%), methanol, potassium fluoride (KF) (99%), sodium acetate trihydrate, acetic acid, anhydrous sodium sulfate and albumin from human resources were obtained from Sigma-Aldrich (St. Louis, MO) and used without purification. Human plasma with sodium citrate added was obtained from Innovative Research (Southfield, MI). Sarin (isopropyl methylphosphonofluoridate) and soman (pinacolyl methylphosphonofluoridate) were obtained from the U.S. Army Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD, USA). Bond Elute C₁₈ 100 mg SPE cartridges (Varian, Harbor City, CA) used to bind the sarin and soman formed by the fluoride reactivation reaction were conditioned by an ethyl acetate/methanol/water wash sequence. An ethyl acetate wash was required to remove residual traces of organic materials in the SPE cartridges that interfered with the gas chromatographic-mass spectrometry (GC-MS) analysis. The recovery of pure agent bound to the SPE cartridge was in the range of 85 to 95%.

Tissue and Animal Plasma

Guinea pig organ tissue, guinea pig blood, and NHP blood were obtained from animals utilized in Institute Animal Care and Use Committee (IACUC) approved protocols. Animals from which tissues were obtained were maintained under an Association for the Assessment and Accreditation of Laboratory Animal Care-International (AAALAC-I) program.

Sample Preparation

Plasma and albumin samples ranging from 0.5 mL to 2.0 mL were treated with twice the volume of KF (0.7 M) in an acetate buffer (1.2 g acetic acid and 136 mg sodium acetate trihydrate per 100 mL water) and the mixture heated for one hr at 40°C. After heating, the sample was centrifuged and the resulting clear supernatant passed over a conditioned SPE C₁₈ cartridge. Following a 0.8 mL water wash, the agents were eluted from the cartridge with 1.2 mL of ethyl acetate and the eluent dried with anhydrous sodium sulfate.

Guinea pig organ tissue samples ranging from one to six g in weight from the kidney, lung, liver and heart were homogenized with a PowerGen (Fisher Scientific, Pittsburgh, PA) in saline solution with final volumes ranging from five to twenty mL. One to two mL of the mixture was then treated with twice the volume of KF (0.7 M) in acetate buffer and the mixture heated for one hr at 40°C. The clear supernatant of the centrifuged mixtures could be used for greater precision in the assay, but analysis of the nonhomogeneous mixture usually gave higher values of reactivated agent. After heating, the mixture was centrifuged, and the supernatant, usually a clear dark green color, was passed over a conditioned SPE C₁₈ cartridge. Following a 0.8 mL water wash, the agents were eluted from the cartridge with 1.2 mL of ethyl acetate and the eluent dried with anhydrous sodium sulfate. The samples were then analyzed by GC-MS.



Instrumentation and Assay for Soman and Sarin

GC-MS separations were performed on an Agilent 6890 gas chromatograph. The GC was fitted with a 30 m \times 0.25 mm I.D. DB-5MS bonded phase column, 0.25 μ m film thickness (J&W Scientific, Folsom, CA). Helium was used as the carrier gas at a column head pressure of 12 psi. The oven temperature was held initially at 50°C for 2 min, programmed from 50 to 100°C at 10°C min⁻¹, and held at 100°C for 3 min. Splitless injections of 1 μ L volume were made using an Agilent 7683 autosampler. Electron ionization MS analyses were performed on an Agilent 5973 mass selective detector interfaced to the GC. The electron ionization MS operating conditions were as follows: ion source pressure approximately 1.5×10^{-5} torr; source temperature, 230°C; electron energy, 70 eV; and electron multiplier voltage +400 V relative to the autotune setting. The mass spectrometer was operated using selected ion monitoring (SIM). Two ions (m/z 99 and 126) for soman and two ions (m/z 99 and 125) for sarin were monitored. A dwell time of 100 ms for each ion resulted in a scan rate of 4.26 cycles sec⁻¹. The retention times for the single peak of sarin ranged from 5.46 to 5.49 min and the two peaks from the diastereomeric mixture of soman ranged from 8.62 to 8.64 and 8.73 to 8.75 min using the conditions described above. The limit of detection with a 2/1 signal to noise ratio was 0.5 pg on column or 0.5 ng/ml in ethyl acetate solvent.

RESULTS

In Vivo Studies with Guinea Pig Plasma and Tissue

Low-Level Subcutaneous Exposure

Reactivation of sarin and soman was performed on guinea pig organ tissues (liver, heart, kidney or lung) and plasma following repeated low-level subcutaneous exposures (0.05, 0.1, 0.2 or 0.4 LD₅₀) of the agent. For the guinea pig 1.0 LD₅₀ sarin

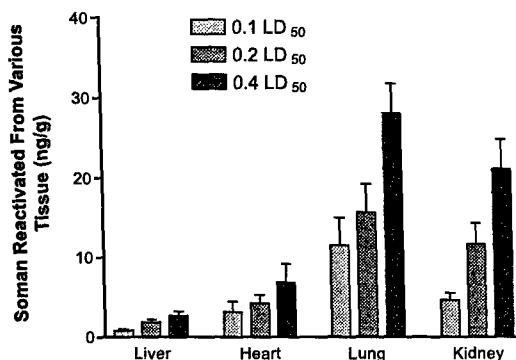


Figure 1. Dose-response values obtained from four different types of guinea pig tissue after five consecutive daily subcutaneous exposures to soman. The animals were sacrificed four hr after the last exposure.

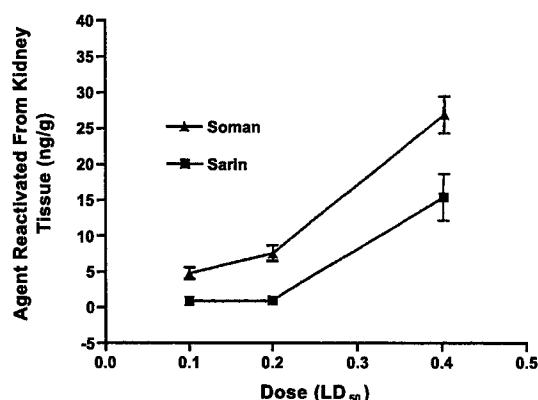


Figure 2. Dose-response values comparing sarin and soman reactivated from guinea pig kidney tissue after repeated daily subcutaneous exposures (10 days with soman and 15 days with sarin). The animals were sacrificed four hr after the last exposure.

equals 42 $\mu\text{g/Kg}$ and 1.0 LD_{50} soman equals 28 $\mu\text{g/Kg}$. Soman was administered once daily for 5 days (Mon–Fri) in Experiment A (data used in Figures 1, 4, and 5) or for 10 days over a 12-day period (Mon–Fri) in Experiments B and C (data used in Figures 2 and 4). Sarin was administered once daily for 15 days (Mon–Fri) over a 19-day period (data used in Figures 2 and 3). Animals were sacrificed at various times following the exposure periods, 4 hr for Exp. A and B, 72 hr for Exp. C, and 2 and 4 hr for the sarin experiment. The variation of soman levels reactivated from heart, kidney, liver or lung tissue following exposure is shown in Figure 1. The error bars represent one standard deviation in the values for six samples analyzed in duplicate. Because kidney and lung tissues were the greatest source of reactivated agent, those tissues were primarily used for subsequent studies. Comparison of sarin and soman reactivated from kidney tissue as a function of dose is depicted in Figure 2. The amount of reactivated soman was greater than the amount of reactivated sarin

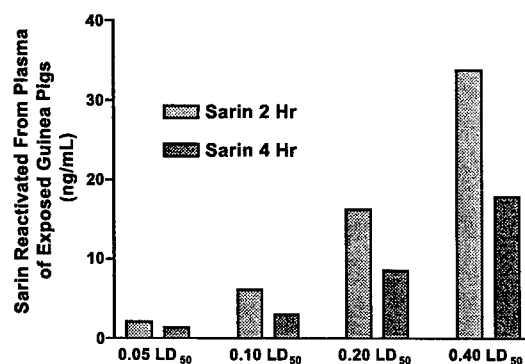


Figure 3. Dose-response values comparing the quantity of reactivated sarin from guinea pig plasma collected either two or four hr after the last of fifteen daily subcutaneous exposures.

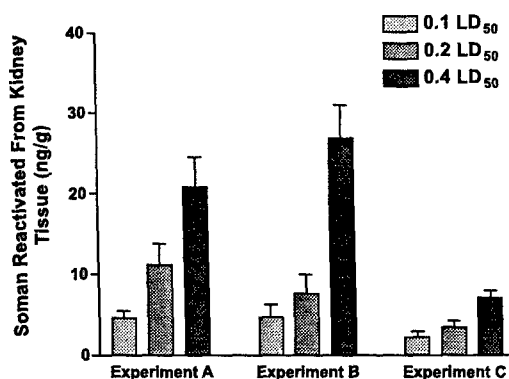


Figure 4. Dose-response values comparing the quantity of reactivated soman obtained from guinea pig kidney tissue exposed under three different experimental conditions. Exp. A involved five consecutive daily exposures with the animals sacrificed four hr after the last exposure. Exp. B involved ten exposures over two weeks with the animals sacrificed four hr after the last exposure. Exp. C involved ten exposures over two weeks with the animals sacrificed 72 hr after the last exposure.

observed at each dose level. In plasma, the impact of post-exposure sacrifice time on reactivated agent level is illustrated in Figure 3. A difference of 2 hr (sacrifice time 2 hr vs. 4 hr post-exposure) resulted in a 50% decrease in the amount of agent reactivated from plasma at the 0.4 LD₅₀ level. Similar trends were observed in the dose-response of soman (0.1, 0.2, 0.4 LD₅₀) reactivated from kidney tissue harvested at 4 hr (Exp. A and B) and at 3 days (Exp. C) post-exposure (Figure 4). The 3-days post-exposure level from the 0.4 LD₅₀ dose was found to be 25% that of the 4-hr post-exposure level. The data in Figure 4 also demonstrated the effect of different dosing schedules on the amount of reactivated agent, with Exp. A using daily doses over a 5-day period while Exp. B involved a total of 10 doses over a 12-day period.

In an attempt to explain the reason for the large storage capacity of the kidney for soman-protein adducts, the fibrous kidney tissue was washed six times with saline, air dried, and tested for reactivated soman. Analysis yielded only an average of 2 ng/g of reactivated soman, a small percentage of the level of close to 30 ng/g for freshly ground tissue.

Comparison of Fluoride Reactivation Values and Plasma BuChE Inhibition Values

Fluoride reactivation of soman values from kidney tissue were compared with plasma BuChE inhibition values,^[2] calculated as a percent of control at 0.1, 0.2, and 0.4 LD₅₀ dose levels. The fluoride reactivation values represent the average of two values for each sample. The relationship with a correlation coefficient of 0.8226 is presented in Figure 5. Note that after an exposure of 0.4 LD₅₀ over 90% of the BuChE was inhibited.

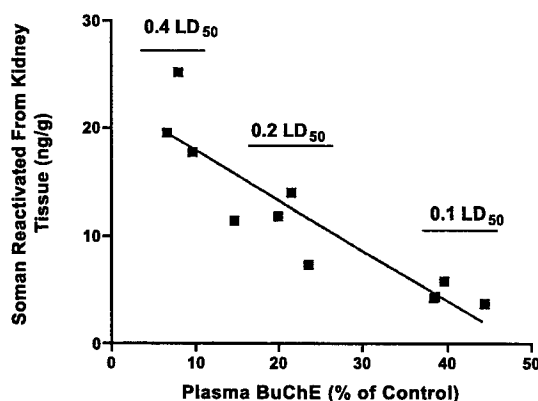


Figure 5. A comparison of reactivated soman values from guinea kidney tissue with plasma BuChE values (% of control) obtained by a modified Ellman method^[2] at three different exposure levels. The correlation coefficient was 0.8226.

High-Dose Dermal Exposure

Guinea pigs were dermally dosed with neat soman at the 18 to 44 LD₅₀ (200 to 500 mg/Kg) level. Dermal decontamination with a proprietary mixture was performed shortly thereafter. Lung and kidney samples were analyzed 24 hr after exposure. The data summarized in Table 1 displayed no clear dose-response relationship. All doses except for the lowest exposure level of 18 LD₅₀ (200 mg/Kg) led to death within 24 hr.

Table 1. Soman reactivated from lung and kidney tissue 24 hr after high level dermal exposure followed by washing of the exposed area with decontaminating solution.^a

Initial dermal exposure ^b	Soman reactivated ^c	
	Lung ng/g	Kidney ng/g
Soman mg/Kg		
200	12.2	13.5
300	20.1	52
350	17.9	41
400	14.4	— ^d
400	18.6	— ^d
450	22.1	53.7
500	16.1	— ^d

^aThe lowest dosed animal survived. Specific expiration times for the other animals were not noted but occurred within 24 hr of exposure.

^bA 1.0 LD₅₀ dose of soman for a dermal exposure is 11.3 mg/Kg for the guinea pig.

^cThe values reported are averages of two samples.

^dThe sample was not available.



Table 2. Soman reactivated from guinea pig tissue harvested 24 hr post-exposure and 24 hr or 6 days postmortem after a 2 LD₅₀ (56 mg/Kg) subcutaneous exposure.

Tissue	Soman reactivated ^a
	ng/g tissue
Blood clot ^b	11
Kidney ^b	147
Liver ^b	23
Heart ^b	18
Lung ^c	26

^aAverage value of two samples.

^b24 hr postmortem and 48 hr after exposure.

^cSix days postmortem and seven days after exposure.

Postmortem Analysis of Clotted Blood and Organ Tissue

Analysis of tissue from two guinea pigs sacrificed 24 hr after a 2 LD₅₀ exposure with both pretreatment (pyridostigmine) and treatment (atropine and 2-PAM) was performed on four types of tissue, clotted blood, kidney, liver and heart, after the carcasses had been left at room temperature for an additional 24 hr. Lung tissue was removed and stored uncovered at room temperature prior to analysis six days later. The data for this experiment are summarized in Table 2.

NHP Blood Analysis

Determination of reactivated soman levels was performed with the plasma and red blood cells of two NHP exposed to a 2 LD₅₀ (15 µg/Kg) intramuscular dose of soman. Both a pretreatment (pyridostigmine) and treatment (atropine, 2-PAM and midazolam) were given to the animals. Plasma values averaging 2.4 ng/mL of reactivated soman were obtained three days after exposure. Lysed red blood cells yielded an average value of 1.0 ng/mL of reactivated soman. On analysis, plasma samples taken 7 days later yielded only a trace (< 0.1 ng/mL) of reactivated soman; however, the lysed red blood cell fraction yielded 0.5 ng/mL of reactivated soman.

In Vitro Studies with Plasma and Albumin

Human plasma and albumin samples were spiked with either sarin or soman dissolved in ethyl acetate solvent, warmed to 40°C, and then analyzed for the reactivated agent at the appropriate time. The samples were filtered over a SPE C₁₈ cartridge to remove any unreacted agent prior to treatment with KF. Values of reactivated agents from plasma and albumin, expressed in both ng/mL and pmol/mL from the 2 µg/mL spiking level are presented in Table 3. Note the values relative to the estimated concentration of BuChE

Table 3. A comparison of sarin and soman concentrations reactivated in human blood components after high dose spiking.

Blood component	Sarin reactivated		Soman reactivated	
	ng/mL	pmol/mL ^a	ng/mL	pmol/mL ^a
Human plasma (2 µg agent/mL plasma) ^b	38	270	132	725
Human albumin ^c (2 µg agent/mL solution)	180	1286	465	2554
Precipitated protein from albumin solution (From 2 µg agent/mL solution)	114 ^d	814 ^e	78 ^d	428 ^e
Commercial human BuChE ^f (0.2 µg agent/mL solution)	1.8	13	4.4	24

^aThe average value of BuChE in human plasma has been reported to be 80 pmol/mL. (From Ref. [19].)

^bAnalyzed 2 hr after mixing.

^cPurified human albumin at a concentration of 30 mg/mL and analyzed 24 hr after mixing.

^dThe dimensional units are ng/30 mg.

^eThe dimensional units are pmol/30 mg.

^fPrepared at a concentration to give a BuChE activity comparable to that in normal human plasma.

(80 pmol/mL) in human plasma.^[19] Figure 6 illustrates the linear relationship between initial spiked concentration and reactivated agent from 0.25 to 2.0 µg/mL. The correlation coefficients for the curves were 0.9292 for sarin and 0.9918 for soman. Each data point on the curve represents the average of six samples with CV's ranging

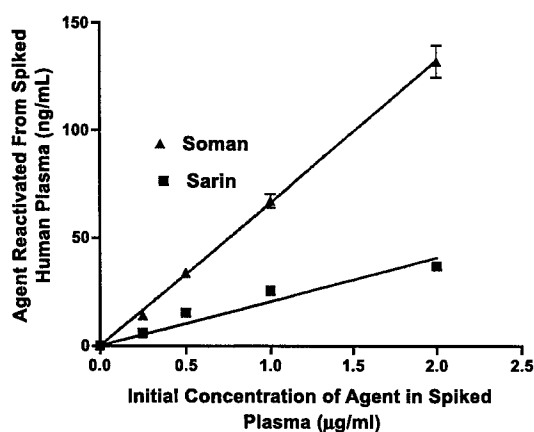
**Figure 6.** Comparison of the quantity of reactivated sarin and soman formed after spiking human plasma samples and heating at 40°C for two hr.

Table 4. The relative stability at 40°C of sarin and soman plasma adducts that formed reactivated agents upon reaction with KF.^a

Time of heating	Reactivated from human plasma	
	Sarin ng/mL	Soman ng/mL
2 hr	4.1	2.5
6 hr	2.7	2.0
24 hr	2.0	1.8
72 hr	1.3	1.3

^aPlasma samples were spiked with either 0.60 µg/mL sarin or 0.78 µg/mL soman diluted in saline. The mixtures were heated at 40°C for the listed times.

from 6 to 9%, and the error bars represent a variation of one standard deviation. Recovery of reactivated agent as a percentage of the initial spiking quantity averaged 1.9% and 6.6% for sarin and soman, respectively, in the plasma exposure and 9.0% and 23.2% for sarin and soman, respectively, in the albumin exposure. Samples of the exposed albumin solutions were precipitated, washed, dried, and analyzed to confirm albumin as a source of reactivated agent. Treating solutions of commercially available BuChE, diluted to normal human plasma levels, with the two nerve agents and subsequent reactivation yielded 1.8 ng/mL for sarin and 4.4 ng/mL for soman.

The data in Table 4 reflect the stability of the agent-protein adducts in plasma after spiking plasma with agents dissolved in saline. Slightly different rates of loss for the sarin- and soman-protein adducts were observed. The concentration of the reactivated sarin decreased 68% and the reactivated soman decreased 47% in three days. The initial concentrations of agent-protein adducts were considerably lower (3.2 ng/mL for soman) when the agents were dissolved in saline rather than ethyl acetate (40 ng/mL for soman) (from Figure 6) prior to the spiking.

DISCUSSION

The fluoride reactivation process was found to be effective for evaluating the relative levels of soman and sarin exposures of guinea pigs. The quantity of reactivated soman and sarin generated by the reactivation process with plasma and organ tissue was measured by electron-impact GC-MS after SPE purification and concentration of the agents. The likely, but not proven, major source of the reactivated agents was the agent adduct of plasma CaE^[13,15-18,20] rather than BuChE.^[7,8] The soman adduct of BuChE ages too rapidly to be a significant source of reactivated soman in this study.^[1,12] Relatively high levels of reactivated agent in kidney and lung tissue compared to heart and liver tissue (Figure 1) were consistent with prior measurements of soman deposition in tissue using radiolabels.^[12] Even though the liver is usually considered the major source of CaE, the plasma CaE was likely to be the major reactant in forming the CaE-agent adducts.^[20-22] The exposure levels of the agents studied ranged from 0.05 LD₅₀ to 2 LD₅₀ sarin or soman delivered by subcutaneous injections, and up to 44 LD₅₀ with dermal exposure.



All repeated low-level (0.05 to 0.4 LD₅₀) exposure experiments led to positive dose-response relationships (Figures 1, 2, 3, and 4) with reactivated agent values ranging from 1.0 ng/g determined in the kidney tissue of the 0.1 LD₅₀ sarin exposure (Figure 2) to 34 ng/(mL) determined in the plasma of the 0.4 LD₅₀ sarin exposure (Figure 3). Large increases of both the reactivated soman and sarin levels in kidney tissue were observed after 10 or 15 repeated doses at the 0.4 LD₅₀ level compared with the 0.2 LD₅₀ level (Figure 2). The reactivated soman level rose from 7 ng/g to 26 ng/g, almost a four-fold increase, and the reactivated sarin level rose from 2 ng/g to 14 ng/g, a seven-fold increase. These increases could be accounted for by greater accumulation of the CaE-agent adduct and other possible adducts in kidney tissue after each exposure at the higher, 0.4 LD₅₀, dose.^[22] At lower doses and fewer repeated exposures a greater portion of the agent-protein adducts may have been transformed or cleared before the next agent dose was given. Reactivated sarin levels in plasma from 0.05 to 0.40 LD₅₀ repeated exposures rose smoothly over the entire range (Figure 3), indicating a less significant accumulation factor in plasma at the 0.4 LD₅₀ exposure level.

The post-exposure analysis time-related variation in concentrations of reactivated agents, attributed to the rapid transforming or clearing of agent adducts in live animals, presented a major potential problem for obtaining accurate dose-response relationships. In the analysis of plasma from sarin exposures (Figure 3), reactivated sarin levels determined from samples obtained 2 hr after exposure were almost twice the values obtained from samples obtained 4 hr after exposure. Another large change with time after exposure in agent reactivation values is illustrated in Figure 4. Both Exp. A and Exp. B samples were collected 4 hr after the last of a series of repeated soman exposures, but Exp. C samples were collected 72 hr after the last of the repeated exposures. Reactivated soman values measured in Exp. C were only one-half to one-fifth of the values measured in Exp. A and B at similar exposure levels. Therefore, for optimal dose-response correlations with this assay, samples should be collected and processed (frozen or analyzed) at similar times after exposure.

The highest level of reactivated soman in this study, 147 ng/g (Table 2), was measured in kidney tissue samples after two drug-protected guinea pigs were exposed to a 2 LD₅₀ dose. While the plasma level of reactivated soman averaged 8.7 ng/mL 24 hr after exposure and one hr postmortem, the average kidney level, 48 hr after exposure and 24 hr postmortem, was seventeen times higher at 147 ng/g. Further examination of the data in Table 2 indicated that 1) the 24 hr postmortem clotted blood level of 11 ng/g was quite similar to the plasma level (8.7 ng/mL) one hr postmortem, 2) the kidney, liver and heart levels (147, 23, and 18 ng/g, respectively) were still relatively high 24 hr postmortem, and 3) the lung tissue value of 26 ng/g was still high seven days postmortem. Those three factors suggested that the rate of clearing of the soman adducts after death must be slow compared with the rate of clearing of soman adducts in the living animal. The slow rate of the decreasing quantity of reactivated soman measured postmortem could be an excellent aid in forensic retrospective analysis by not only verifying the particular agent involved in an exposure but also affording some indication of the time of the exposure.

The quantity of reactivated soman from kidney tissue was correlated (coefficient = 0.8226) with the plasma BuChE level (Figure 5) determined by a modified Ellman Colorimetric Method that has become a standard method for determining the magnitude of nerve agent exposure.^[2,23] The inverse trend over the exposure range



from 0.1 to 0.4 LD₅₀ reveals a major deficiency of the plasma BuChE method. Because the level of active BuChE was already below 10% of the control level at the 0.4 LD₅₀ exposure level, the effect of higher agent exposures would not be measured accurately and differentiation between higher levels of exposure would be unlikely. Reactivated agent values can continue to rise to higher values as seen in the 2 LD₅₀ data described above, where reactivation levels rose to 147 ng/g with continued quantitative precision. Therefore, the fluoride reactivation reaction could become a useful method for more accurate measurement of much higher levels of nerve agent exposures particularly in the cases of pre-treated or treated animals where survival continues after very high exposure levels.

The analysis of lung and kidney tissue from guinea pigs given 18 to 44 LD₅₀ (200 to 500 mg/Kg) dermal applications of neat soman prior to attempted decontamination showed no particular dose- or exposure-related response trend (Table 1). One animal with the lowest dose, 18 LD₅₀, survived for 24 hr, and postmortem analysis yielded the lowest reactivated soman levels in both lung (12.2 ng/g) and kidney (13.5 ng/g). Reactivated soman levels for the other seven animals (27 to 44 LD₅₀) ranged from 14.4 to 22.1 ng/g for lung tissue. Kidney reactivated soman levels for three animals ranged from 41 to 53.7 ng/g. The leveling effect observed in the analysis of both types of tissue reflected both the success of the decontamination effort and the range of soman levels at which the exposure was lethal without the aid of a pretreatment or treatment.

In primates, previously reported studies^[7,8] indicated that the major source of reactivated sarin came from sarin-ChE adducts. In our study soman was reactivated in the plasma (2.4 ng/ml) and red blood cells (1.2 ng/ml) of two NHP 3 days after a 2 LD₅₀ exposure. Because soman-BuChE adducts age rapidly^[11,12] and CaE does not circulate in primate blood at any significant level, there must be another source of reactivated soman.

The tyrosine group in albumin has been shown to form an adduct with soman^[14] which could be the source of the reactivated soman. In this study, human plasma proteins including albumin were shown to have a significant capacity to bind both soman and sarin (Table 3) when the agents were dissolved in ethyl acetate prior to spiking. The presence of ethyl acetate appears to decrease the rate of hydrolysis of the agents by hydrolases.^[20,24] For example, at a spiked level of 2 µg soman per mL of human plasma, the reactivated soman level was found to be 725 pmol/mL, nine times the theoretical quantity (80 pmol/mL)^[19] of human plasma BuChE in the same volume of plasma. The relationship between the quantity of spiked soman in plasma and reactivated soman was linear over the range of 0.25 to 2 µg/ml (Figure 6) indicating that binding sites of the proteins were not saturated at those concentrations. Treatment of albumin in saline with 2.0 µg/ml soman gave 2554 pmol/ml of reactivated soman that was thirty two times the 80 pmol/ml quantity. Precipitation and analysis of the albumin from the above reactions yielded reactivated agent values relative to the quantity of albumin recovered (Table 3) indicating that the albumin was indeed the major source of the reactivated agents.

Spiking with excess soman or sarin dissolved in ethyl acetate of protein with BuChE at activity equivalent to normal average BuChE levels in human plasma yielded reactivated agent quantities for both sarin and soman relative to the total concentration of protein rather than to BuChE activity (Table 3). This experiment gave additional

support to the concept that reactivated soman and sarin could be derived from sources of proteins such as albumin in addition to the cholinesterases in primates.

The change in concentration of the reactivated agents in human plasma with respect to time (Table 4) gave insight into the stability of the agent-protein adducts. In three days the reactivated sarin level decreased 67% and the reactivated soman level decreased 47%. The decrease in the NHP reactivated soman value of 2.4 ng/mL at day three to a trace value at day ten was reasonably consistent with the *in vitro* determined rate of decreasing concentration and also gave additional support to the hypothesis that the reactivated soman could have been generated from the soman-albumin adduct.

Even though the rate of reaction of BuChE with the soman and sarin is far greater than the rate of reaction of albumin with the nerve agents, once sarin or soman binds sufficient BuChE, the reaction with the highly abundant albumin in plasma should increase in quantity. Certainly, after a 2 LD₅₀ exposure leading to extremely low reactive BuChE levels, a measurable reaction with albumin would become more likely. Further study is proceeding to more clearly discern the sources of the reactivated agents.

In summary, the fluoride reactivation process to evaluate soman and sarin nerve agent exposures in guinea pigs was specific, precise, and sensitive. Because the source of reactivated agent was cleared rapidly from the blood and tissue of live animals, dose-response correlations could have considerable error unless samples are removed and processed at similar times after exposures. The slower clearing of the agent-protein adduct after death would be advantageous for forensic analysis. The preliminary work of reactivating soman from the plasma of exposed NHPs and human plasma and albumin suggests that the reactivation process could also be used for evaluating soman exposures to humans and other animals.

ACKNOWLEDGMENTS

We greatly appreciate the biological samples shared by Dr. John McDonough, Dr. Tony Shih, Maj. Maurice Sipos (Ph.D.), Dr. Robert Sheridan, Dr. Edward Clarkson, and Dr. Melinda Roberson from their approved animal-use protocols. We would also like to thank LTC Harry Slife (Ph.D.), Shannon Shuman, Connie Clark, Anita Moran, Chris Byers, Evan Holloway, Megan Grahm, Bethany Reynolds, and Wes Holmes for their technical assistance in portions of this study. W. Korte is grateful for the generous funding from the Department of Defense, Defense Threat Reduction Agency, under the auspices of the U.S. Army Research Office Scientific Services Program administered by Battelle (Delivery Order 802, Contract No. DAAHO4-96-C-0086).

REFERENCES

1. Noort, D.; Benschop, H.P.; Black, R.M. Biomonitoring of exposure to chemical warfare agents: a review. *Toxicol. Appl. Pharmacol.* **2002**, *184* (2), 116–126.
2. Nostrandt, A.C.; Duncan, J.A.; Padilla, S. A modified spectrophotometric method appropriate for measuring cholinesterase activity in tissue from carbaryl-treated animals. *Fundam. Appl. Toxicol.* **1993**, *21* (2), 196–203.



3. Benschop, H.P.; Bijleveld, E.C.; Otto, M.F.; Degenhardt, C.E.A.; Van Helden, H.P.M.; De Jong, L.P.A. Stabilization and gas chromatographic analysis of the four stereoisomers of 1,2,3-trimethylpropyl methylphosphonofluoridate (soman) in rat blood. *Anal. Biochem.* **1985**, *151* (2), 242–253.
4. Shih, M.L.; Smith, J.R.; McMonagle, J.D.; Dolzine, T.W.; Gresham, V.C. Detection of metabolites of toxic alkylmethylphosphonic acids in biological samples. *Biol. Mass Spectrom.* **1991**, *20* (11), 717–723.
5. Shih, M.L.; McMonagle, J.D.; Dolzine, T.W.; Gresham, V.C. Metabolite pharmacokinetics of soman, sarin, and GF in rats and biological monitoring of exposure to toxic organophosphorus agents. *J. Appl. Toxicol.* **1994**, *14* (3), 195–199.
6. Driskell, W.J.; Shih, M.; Needham, L.L.; Barr, D.B. Quantitation of organophosphorus nerve agent metabolites in human urine using isotope dilution gas chromatography-tandem mass spectrometry. *J. Anal. Toxicol.* **2002**, *26* (1), 6–10.
7. Polhuijs, M.; Langenberg, J.P.; Noort, D.; Hulst, A.G.; Benschop, H.P. Retrospective detection of exposure to organophosphates: analyses in blood of human beings and rhesus monkeys. In *NBC Risks*; Sohns, T., Voicu, V.A., Eds.; Kluwer Academic Publishers: Netherlands, 1999; 513–521. Presented by De Jong, D.P.A.
8. Polhuijs, M.; Langenberg, J.P.; Benschop, H.P. New method for retrospective detection of exposure to organophosphorus anticholinesterases: application to alleged sarin victims of Japanese terrorists. *Toxicol. Appl. Pharmacol.* **1997**, *146* (1), 156–161.
9. Jakubowski, E.M.; Heykamp, L.S.; Durst, H.D.; Thomson, S.A. Preliminary studies in the formation of ethyl methylphosphonofluoridate from rat and human serum exposed to VX and treated with fluoride ion. *Anal. Lett.* **2001**, *34* (5), 727–737.
10. Fidler, A.; Noort, D.; Hulst, A.G.; De Ruiter, R.; Van der Schans, M.J.; Benschop, H.P.; Langenberg, J.P. Retrospective detection of exposure to organophosphorus anti-cholinesterases: mass spectrometric analysis of phosphorylated human butyrylcholinesterase. *Chem. Res. Toxicol.* **2002**, *15* (4), 582–590.
11. Barak, R.; Ordentlich, A.; Barak, D.; Fischer, M.; Benschop, H.P.; De Jong, L.P.A.; Segal, Y.; Velan, B.; Shafferman, A. Direct determination of the chemical composition of acetylcholinesterase phosphorylation products utilizing electrospray-ionization mass spectrometry. *FEBS Lett.* **1997**, *407* (3), 347–352.
12. Fleisher, J.H.; Harris, L.W. Dealkylation as a mechanism for aging of cholinesterase after poisoning with pinacolyl methylphosphonofluoridate. *Biochem. Pharmacol.* **1965**, *14* (5), 641–650.
13. De Jong, L.P.A.; Van Dijk, C. Formation of soman (1,2,2-trimethylpropyl-methylphosphonofluoridate) via fluoride-induced reactivation of soman-inhibited aliesterase in rat plasma. *Biochem. Pharmacol.* **1984**, *33* (7), 663–669.
14. Black, R.M.; Harrison, J.M.; Reed, R.W. The interaction of sarin and soman with plasma proteins: the identification of a novel phosphorylation site. *Arch. Toxicol.* **1999**, *73* (2), 123–126.
15. Maxwell, D.M.; Brecht, K.M. Carboxylesterase: specificity and spontaneous reactivation of an endogenous scavenger for organophosphorus compounds. *J. Appl. Toxicol.* **2001**, *21* (Suppl. 1), S103–S107.
16. Clement, J.G. Importance of aliesterase as a detoxification mechanism for soman



- (pinacolyl methylphosphonofluoridate) in mice. *Biochem. Pharmacol.* **1984**, 33 (23), 3807–3811.
17. Clement, J.G.; Benschop, H.P.; De Jong, L.P.A.; Wolthuis, O.L. Stereoisomers of soman (pinacolyl methylphosphonofluoridate): inhibition of serum carboxylic ester hydrolase and potentiation of their toxicity by CBDP (2-2-methylphenoxy)-4H-1,3,2-benzodioxaphosphorin-2-oxide) in mice. *Toxicol. Appl. Pharmacol.* **1987**, 89 (1), 141–143.
 18. Maxwell, D.M.; Brecht, K.M.; O'Neill, B.L. The effect of carboxylesterase inhibition on interspecies differences in soman toxicity. *Toxicol. Lett.* **1987**, 39 (1), 35–42.
 19. Myers, D.K. Studies on cholinesterase 7: determination of the molar concentration of pseudo-cholinesterase in serum. *Biochem. J.* **1952**, 51, 303–311.
 20. Maxwell, D.M.; Vlahacos, C.P.; Lenz, D.E. A pharmacodynamic model for soman in the rat. *Toxicol. Lett.* **1988**, 43 (1–3), 175–188.
 21. Clement, J.G. Role of aliesterase in organophosphate poisoning. *Fundam. Appl. Toxicol.* **1984**, 4 (2 Pt. 2), S95–S105.
 22. Sterri, S.H.; Lyngaas, S.; Fonnum, F. Toxicity of soman after repetitive injection of sublethal doses in guinea pig and mouse. *Acta Pharmacol. Toxicol.* **1981**, 49 (1), 8–13.
 23. Worek, F.; Mast, U.; Kiderlen, D.; Diepold, C.; Eyer, P. Improved determination of acetylcholinesterase activity in human blood. *Clin. Chim. Acta* **1999**, 288 (1–2), 73–90.
 24. Gay, D.D.; Hoskins, F.C.G. Stereospecificity and active site requirements in a diisopropylphosphorofluoridate-hydrolyzing enzyme. *Biochem. Pharmacol.* **1979**, 28 (7), 1259–1261.

